NOVEL ANTITUMOR ANTIBIOTICS, CI-940 (PD 114,720) AND PD 114,721*

TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

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The novel, broad-spectrum antitumor antibiotics, CI-940 (PD 114,720) and PD 114,721 are components of bioactive compounds produced by a novel actinomycete (ATCC 39366). The organism is unique in that whole cell analysis revealed LL-diaminopimelic acid and major amounts of arabinose.

These new antibiotics exhibit potent antitumor activity *in vitro* and *in vivo versus* an array of tumors and possess strong antibacterial and antifungal activity.

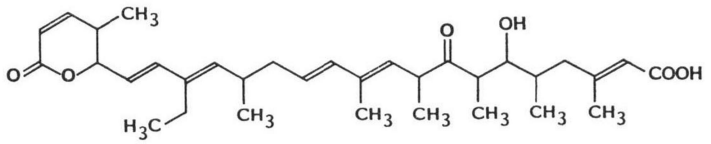
The above antibiotics are produced in 7,570-liter fermentors at yields of 5~8 μg/ml.

During the course of our antitumor antibiotic drug discovery program, the fermentation broth of actinomycete isolate WP-2053 was found active vs. L1210 murine leukemic cells *in vitro*. The producing organism was identified as a novel actinomycete and deposited with the American Type Culture Collection (Rockville, Md) as ATCC 39366.

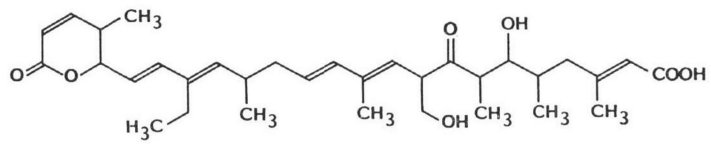
The biological activity found in the fermentation broth was attributed to a complex of compounds. The two major components of the complex were isolated and identified as CI-940 (PD 114,720) and PD 114,721 (Fig. 1). Structural studies on these two compounds were carried out by SCHAUMBERG *et al.*[1].

This paper describes the taxonomy of the producing organism and fermentation and biological aspects of the two compounds.

Fig. 1. Structure of PD 114,720 (A) and PD 114,721 (B).



A



B

* The provisional names, elactocin and hydroxyelactocin, have been assigned, respectively to CI-940 (PD 114,720) and PD 114,721.

Materials and Methods

Culture Isolation and Characterization

The culture was isolated from a soil sample collected in East Greenville, PA. The plating medium consisted of 3% glycerol, 0.25% L-asparagine, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4 and 1.5% agar.

Culture characterization was carried out following the ISP (International Streptomyces Project) procedure[2]. The culture was maintained on Amidex Corn Starch Agar ADX[3] slants at 24°C. Morphological and color determinations of the growth of the organism were made at weekly intervals over a six-week period. Purified cell walls and whole cell hydrolysates were analyzed following the methods of BECKER *et al.*[4] and LECHEVALIER[8], respectively.

Fermentation

Stock cultures of the organism were maintained in lyophilized vials, and working cultures stored as cryovials in liquid nitrogen refrigerator (Union Carbide, Indianapolis). To start a fermentation, the contents of a thawed cryovial was used to inoculate a 300-ml seed flask containing 50 ml of seed medium. The inoculated flask was incubated with shaking (New Brunswick Shaker, 5-cm throw) at 24°C for 2~3 days. The seed medium consisted of: 0.5% Amberex 1003 yeast (Amber Labs.), 0.1% glucose monohydrate, 2.4% dextrin Amidex B411 (Corn Products), 0.5% N-Z case (Humko-Sheffield), 0.3% spray-dried meat solubles (Daylin Labs.) and 0.2% $CaCO_3$.

The production of the antitumor complex was carried out in 250-ml Erlenmeyer flasks, and 30- and 7,570-liter fermentors. The fermentation conditions were as follows: 50-ml production medium/250-ml flask, 200 rpm shaker (Model G-53, New Brunswick); 30-liter stirred jar fermentor, 16 liters/jar, 1.0 v/v/minute air, 300 rpm; 760-liter fermentor, 600 liters/tank, 0.75 v/v/minute air (450 liters air/minute), 155 rpm; 7,570-liter fermentor, 4,920 liters/tank, 0.75 v/v/minute air (3,680 liters air/minute), 125 rpm. The fermentations were carried out for 4~6 days at 24°C.

Assay

The antitumor complex in the fermentation broth was assayed both by high pressure liquid chromatography (HPLC) and activity vs. L1210 murine leukemic cells (Mason Res. Inst., Worchester, Mass.) in tissue culture.

For HPLC assay, 5-ml of fermentation broth was acidified with 0.15 ml of 1 N HCl; 5 ml EtOAc was added, mixed thoroughly, then centrifuged for 10 minutes $450 \times g$ (International Clinical Centrifuge, Boston, Mass.). The EtOAc layer was recovered, reduced to dryness then reconstituted in 0.5 ml methanol; 10 μl was chromatographed over a μBondapak C_{18} silica gel column (Waters Associates, Milford, Mass.), at a flow rate of 1.5 ml/minute using 0.05 M ammonium acetate buffer (pH 6.5) - acetonitrile (45: 55) solvent system. The retention time for PD 114,720 was 5.45 minutes and 4.0 minutes for PD 114,721.

The *in vivo* antitumor activity was evaluated vs. P388 murine lymphocytic leukemic and L1210 murine lymphoid leukemia tumor cell lines in CDF mice[5]. The tumor cells were injected intraperitoneally (ip) on day 0 and the compounds were also introduced ip on days 1~9.

Results

Morphological and Cultural Characteristics

Isolate WP-2053 was identified as a member of the Grey series of actinomycetes, Table 1. The spores were produced in a spiral chain with 10 or more spores in a chain (Fig. 2). The spores were smooth and cylindrical or rectangular in shape (Fig. 3).

Cell Wall and Whole Cell Sugar Analysis

The cell wall of isolate WP-2053 contained LL-diaminopimelic acid, and glycine which are characteristic of type I cell wall. A unique feature of the organism was the presence of a major amount of

Table 1. Mycelial and substrate color and sporulation of isolate WP-2053.

Medium		Color*	Spore chain
Yeast extract - malt extract agar	AM*	Slate gray (13 ih)	Spiral(s)
(ISP No. 2)	R	Mustard gold (2 pg)	
	SP	None	
Oatmeal agar	AM	None	Spiral(s)
(ISP No. 3)	R	Olive (2 pl)	
	SP	None	
Inorganic salts - starch agar	AM	Pewter gray (13 fc)	Spiral(s)
(ISP No. 4)	R	Colorless	
	SP	None	
Glycerol - asparagine agar	AM	Near gray (7 ml)	Spiral(s)
(ISP No. 5)	R	Light wheat (2 ea)	
	SP	None	

* Color designation from Color Harmony Manual, 4th Ed., Container Corporation of America, 1958.
Color: AM, aerial mycelium; R, reverse substrate mycelium; SP, soluble pigment.

Fig. 2. Spiral spore chain of isolate WP-2053 (×400).

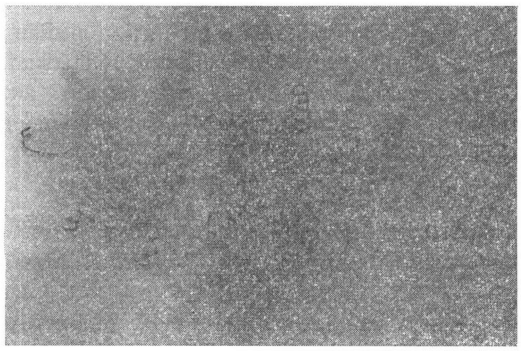
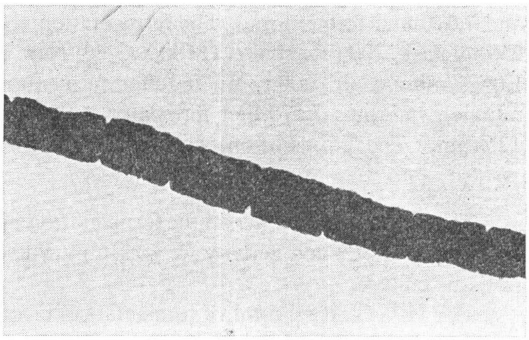


Fig. 3. Chain of cylindrical spores of WP-2053 (×41,300).



arabinose on whole cell analysis.

Physiological Characteristics

The isolate was found to reduce nitrate, liquefy gelatin, and peptonize skim milk. Melanin or other soluble pigments were not formed. The culture utilized 10 of the 16 carbons tested; it did not utilize arabinose, inulin, lactose, maltose, mannitol and sucrose (Table 2).

Antitumor Activity

PD 114,720 and PD 114,721 showed excellent activities vs. L1210 cell line *in vitro*: ID_{50} of 0.12 and 0.19 ng/ml, respectively. The activities vs. L1210 and P388 murine leukemias *in vivo* are shown in Table 3. The bulk of our data, not reported here, indicates that doses $\geq$0.1 mg/kg are toxic in the described treatment schedule. A detailed description of the antitumor activities in other tumor systems is reported in a separate communication[8].

Antimicrobial Activity

The compounds were both active vs. bacteria and fungi (Table 4). PD 114,720 and PD 114,721 showed the same pattern of activity, although PD 114,721 was overall less potent.

Table 2. Cultural characteristics of isolate WP-2053.

Melanin production on	
Tryptone - yeast extract broth (ISP No. 1)	Negative
Peptone - yeast extract (ISP No. 6)	Negative
Tyrosine agar (ISP No. 7)	Negative
Gelatin liquefaction	Positive
Skim milk-coagulation	Negative
Skim milk-peptonization	Positive
Nitrate reduction	Positive
Carbon utilization*	
L-Arabinose	−
D-Fructose	+
D-Galactose	+
D-Glucose	+
Glycerol	+
i-Inositol	+
Inulin	−
Lactose	−
Maltose	−
D-Mannitol	−
D-Mannose	+
Raffinose	+
Rhamnose	+
Salicin	+
Sucrose	−
D-Xylose	+
Control (no carbon)	−

* −, No growth; +, good growth.

Table 3. *In vivo* antitumor activity of PD 114,720 and PD 114,721 vs. P388 lymphocytic and L1210 lymphoid leukemias.[a]

Tumor system	Dosage[c]	T/C (%)[b]	
		PD114,720	PD114,721
P388	0.0125	137	111
	0.02	141	139
	0.05	148	143
L1210	0.0062	120	120
	0.0125	119	131
	0.02	120	132
	0.05	128	142

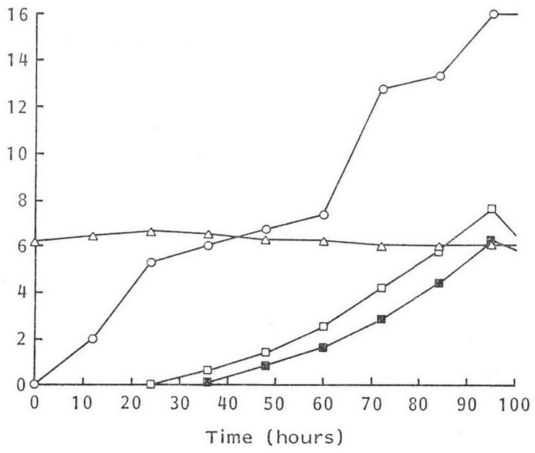
[a] Data provided by NCI (Drug Evaluation Branch, Division of Cancer Treatment).

[b] T/C is the quotient (expressed in %) of the survival time of treated animals (T), and the survival time of control animals (C). T/C values of >125 and >130 for L1210 and P388, respectively, are considered active. Data provided by NCI.

[c] Dosage: mg/kg/injection, daily×9 (ip).

Fig. 4. Production of PD 114,720 and PD 114,721 by fermentation of isolate WP-2053.

△ pH, ○ sedimentation (growth, %), □ PD 114,720 (μg/ml), ■ PD 114,721 (μg/ml).



Fermentation

The initial shake flask medium (SF) which gave trace amount of the complex consisted of 1.5% sucrose, 1.0% lactose, 0.65% peptonized milk (Humko-Sheffield, Ill.), 0.35% fish meal (Zapata Haynie, Md.), and 0.25% torula yeast (Lake States, Wis.). The fermentation broth at a dilution of 1: 1,000 inhibited 98% of L1210 growth *in vitro*.

Fermentation development was subsequently carried out and a final production medium (PM) consisting of the following ingredients was developed: 1.5% maltose, 1.0% glucose, 0.75% Pharmamedia, 0.4% corn meal and 0.25% torula yeast.

As shown in Table 5, the production of PD 114,720 and PD 114,721 was higher in the PM than in the SF medium. The production of the antibiotics started about 24 hours after inoculation and peak yields were obtained after 96 hours (Fig. 4). The yields of PD 114,720 and PD 114,721 in the 7,570-liter fermentor were 7.6 and 6.2 μg/ml, respectively.

Table 4. Antimicrobial activity of PD 114,720 vs. PD 114,721.

Test organism	MIC (μg/ml)*	
	PD 114,720	PD 114,721
Escherichia coli 0483	>1,000	>1,000
Salmonella typhimurium TA1535	>1,000	>1,000
Alcaligenes viscolactis 21698	<0.46	37
Branhamella catarrhalis 03596	4.1	12.3
Pseudomonas aeruginosa 05111	>1,000	>1,000
Micrococcus luteus 05064	<0.46	37
Staphylococcus aureus 02482	<0.46	37
Streptococcus pyogenes C203	<0.46	37
S. pneumoniae SV1	<0.46	12.3
S. faecalis 05045	1.4	111
Bacillus cereus 04810	<0.46	37
B. megaterium 066	1.4	37
Saccharomyces cerevisiae S288	333	333
Schizosaccharomyces pombe M1388	<0.46	<0.46
Rhodotorula aurantiaca M1508	12.3	37
Torulopsis albida M1390	>1,000	>1,000
Mucor parasiticus M2652	<0.46	<0.46
Rhizopus japonicus M1557	<0.46	<0.46

* Broth dilution method.

Table 5. Production of PD 114,720 and PD 114,721 in shake-flasks, and 30-liter stirred jars using the SF and PM media.

Media	Yield (μg/ml)			
	Shake-flask		Stirred jar	
	PD 114,720	PD 114,721	PD 114,720	PD 114,721
SF	0.38	0.03	0.09	0.002
PM	5.73	—	2.03	—

Discussion

The culture characteristics of isolate WP-2053 resemble that of chemotype 1 actinomycetes, particularly the *Streptomyces*. However, whole cell analysis revealed the presence of a significant level of arabinose in the cell hydrolysate which makes this organism unique. On this basis, we propose that this organism be classified under a new genus *Elactomyces*. The proposed name for the organism is *Elactomyces cylindrosporae* which will be communicated elsewhere. The culture characteristics of the organism that produce the leptomycins[7], compounds with structural resemblance to PD 114,720 and PD 114,721, were examined and found to be different from WP-2053. Besides the presence of arabinose in the whole cell hydrolysate, isolate WP-2053 differs from the leptomycin-producing culture in that it reduces nitrate, liquefies gelatin and it has a different carbon utilization profile.

PD 114,720 and PD 114,721 possess good antitumor activities vs. an array of murine leukemias and solid tumors[6]. Moreover, these compounds have excellent antimicrobial activities which correlate with the tissue culture activity. The microorganism *Schizosaccharomyces pombe* was particularly used to assay the antibiotic complex in the broth during fermentation development, but HPLC was a more quantitative assay for the two antibiotics.

The antibiotics are apparently excreted into the fermentation medium. Production starts, after the organism has attained sufficient growth, typically 24 hours into the fermentation.

Addendum from the Editorial Office

Kazusamycin (I. UMEZAWA, *et al.*, J. Antibiotics 37: 706~711, 1984; 38: 220~223, 1985) has been reported to have the same structure as PD 114,721.

Acknowledgments

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